# Determination of short-chain fatty acids by *N, N*-dimethylethylenediamine derivatization combined with liquid-chromatography/mass spectrometry and their implication in influenza virus infection

Divyavani Gowda [1#], Yonghan Li [2#], Siddabasave Gowda B. Gowda [1,3], Marumi Ohno [4], Hitoshi Chiba [5], and Shu-Ping Hui[1*]

1. Faculty of Health Sciences, Hokkaido University, Kita-12 Nishi-5, Kita-Ku, Sapporo 060-0812, Japan
2. Graduate school of Health Science, Hokkaido University, Kita-12, Nishi-5, Kita-ku, Sapporo 060-0812, Japan
3. Graduate School of Global Food Resources, Hokkaido University, Kita-12 Nishi-5, Kita-Ku, Sapporo 060-0812, Japan
4. International Institute for Zoonosis Control, Hokkaido University, Kita 20, Nishi 10, Kita-ku, Sapporo, 001-0020, Japan
5. Department of Nutrition, Sapporo University of Health Sciences, Nakanuma Nishi-4-3-1-15, Higashi-Ku, Sapporo 007-0894, Japan

# Equally contributing author.

***Correspondence:**

Prof. Shu-Ping Hui

Faculty of Health Sciences, Hokkaido University, Kita-12 Nishi-5, Kita-Ku, Sapporo 060-0812, Japan ORCID: 0000-0001-9973-6461.

e-mail address: keino@hs.hokudai.ac.jp

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## Abstract

Short-chain fatty acids (SCFAs) are the end products of the fermentation of complex carbohydrates by the gut microbiota. Although SCFAs are recognized as important markers to elucidate the link between gut health and disease, it has been difficult to analyze SCFAs with mass spectrometry technologies due to their poor ionization efficiency and high volatility. Here, we present a novel and sensitive method for the quantification of SCFAs, including C2–C6 SCFAs and their hydroxy derivatives, by liquid chromatography/tandem mass spectrometry (LC-MS/MS) upon *N, N*-dimethylethylenediamine (DMED)-derivatization with a run time of 10 min. Moreover, the quantification method of DMED-derivatized SCFAs in intestinal contents using isotope-labeled internal standards was also established. The method validation was performed by analyzing spiked intestinal samples; the limits of detection and quantification of SCFAs with this method were found to be 0.5 and 5 fmol, respectively; the recovery was greater than 80% and good linearity (0.9932 to 0.9979) of calibration curves was obtained over the range from 0.005 to 5000 pmol/μL; the intra-day and inter-day precision were achieved in the range of 1–5%. Furthermore, the validated method was applied to analyze SCFAs in the cecum and colon contents of mice infected with the influenza virus. The results showed that the concentration of most of the SCFAs tested here decreased significantly in a time-dependent manner after the infection, suggesting a possibility that SCFAs in intestinal samples could be used as severe disease markers. Overall, we here successfully developed a simple, fast, and sensitive method for SCFA analysis by LC-MS/MS combined with DMED-derivatization. The method for the quantification of SCFAs will be a useful tool for both basic research and clinical studies.

## 1. Introduction

Gut microbial metabolites such as short-chain fatty acids (SCFAs) are produced by the fermentation of dietary fiber and have important functions in the modification of pathological and physiological processes [1]. SCFAs are highly volatile fatty acids that consist of one to six carbons, with acetic, propionic, and butyric acids accounting for 90-95% of the total [2]. SCFAs produced in the gut reach the circulation via the portal vein and significantly alter the host energy metabolism not only in the gut but also in other tissues [3]. In addition to energy metabolism, both *in vitro* and *in vivo* studies have demonstrated the anti-inflammatory and anti-tumor properties of the SCFAs [4]. For example, SCFAs inhibit lipopolysaccharide- and cytokine-induced inflammatory responses via activation of G-protein coupled receptors (GPR41 and GPR43) and inhibition of histone deacetylases [5]. SCFAs regulate innate immune cells, regulatory T cells, and an increase in their biosynthesis are linked to preventing the progression of type 2 diabetes and nonalcoholic fatty liver disease [6, 7]. Therefore, the analysis of SCFAs is important for understanding their roles in complex biological systems.

Despite the importance of SCFAs in human health and disease, there is a limited number of sensitive analytical methods are reported. SCFAs are often derivatized and analyzed by conventional techniques such as gas chromatography-mass spectrometry (GC-MS) [8, 9] and high-performance liquid chromatography-ultraviolet detection (HPLC-UV) [10], nuclear magnetic resonance (NMR) [11], capillary electrophoresis [12], and stable isotope tracer technique [13]. However, these techniques have problems such as low sensitivity, the requirement of a large amount of sample, and time-consuming. On the other hand, liquid chromatography-mass spectrometry (LC-MS) has considerable advantages in terms of its high sensitivity, minimal sample requirement, and rapid performance. However, direct analysis of SCFAs by LC-MS is difficult because of their low mass range in mass spectra, insufficient ionization in electrospray ionization, poor chromatographic separation, and the need for harsh experimental conditions such as a highly acidic mobile phase. Hence, SCFAs are often quantitated by LC-MS by derivatizing with various reagents such as 3-nitrophenylhydrazine [14, 15], O-

benzylhydroxylamine [16], $^{12}C$ or $^{13}C$ labeled aniline [17], and 4-acetamido-7-mercapto-2,1,3-benoxadiazole[18]. However, these methods are time-consuming or require drastic conditions such as high temperature to derivatize SCFAs, which could result in evaporation and degradation of SCFAs during the procedures. Recently, *N, N*-dimethylethylenediamine (DMED) reagent is widely applied for the derivatization of fatty acids and their analogs at the carboxylic acid moiety [19–21], which results in the enhancement of detection sensitivity by several folds. In the present study, we aimed to develop a simple and sensitive analytical method for the quantification of SCFAs and their hydroxy derivatives (HSCFAs) in intestinal contents of mice using DMED as a derivatizing agent under mild conditions. The derivatization reaction was optimized, and the analytical validation was performed using standard solutions and a fecal matrix. The method was successfully applied to determine the concentrations of SCFAs and HSCFAs in the colon and cecum contents of mice, which further provide the lipid profile in a severe influenza mouse model.

## 2. Materials and Method

### 2.1 Materials

Triethylamine (TEA), *N, N*-Dimethlyethylenediamine (DMED), 3-hydroxybutyric acid (3HC4), and acetic acid-d4 (C2-d4), were obtained from Sigma-Aldrich (St. Louis, MO, USA). 2-chloro-1-methyl pyridinium iodide (CMPI), propionic acid (C3), butyric acid (C4), valeric acid (C5), and hexanoic acid (C6) were purchased from Tokyo Chemical Industry Co. LTD., (Tokyo, Japan). Acetic acid (C2) and 1M ammonium acetate solution were obtained from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). LC-MS grade methanol and acetonitrile were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). 3-hydroxypropionic acid (3HC3), 4-hydroxy pentanoic acid (4HC5), 3-hydroxyhexanoic acid (3HC6), and pentanoic acid-d9 (C5-d9) were obtained from Cayman Chemical (Ann Arbor, MI, USA). All the three reaction reagents (TEA, DMED, and CMPI) were dissolved in the LC-MS grade acetonitrile at a concentration of 2 mM and stored in a −28°C freezer.

### 2.2 Animal samples

As reported previously [22], influenza virus A/Puerto Rico/8/34 (H1N1; PR8) was obtained from the National Institute of Infectious Diseases, Japan. The virus was propagated in 10-day-old embryonated chicken eggs at 35°C for 48 h, and aliquots of collected allantoic fluids were stored at −80°C. Male C57BL/6 mice were purchased from Hokudo (Sapporo, Japan) and were kept at a BSL-2 laboratory (temperature 22°C ± 2°C, relative humidity 50% ± 10%) at the International Institute for Zoonosis Control, Hokkaido University. The mice were fed with a standard CE-2 chow diet (CLEA, Sapporo, Japan) with water *ad libitum*. Experiments were performed on 11–12 week-old mice. PR8 virus at 500 plaque-forming units (PFU) in 50 µL of PBS or PBS only (control) were intranasally inoculated into the mice under inhalation anaesthesia with isoflurane. On 1, 3, or 6 days post-infection (dpi), the mice were euthanized, and their colon and cecum contents were collected into a 1.5-mL Eppendorf tube (n=5 mice in each group). Each cecum and colon contents were weighed and homogenized (30 sec × 2 cycles) in 10 volumes of acetonitrile with 5–6 ceramic beads (1.4 mm, Cat. No. 15-340-159, Fisherbrand, Pittsburgh, PA, USA), using a BeadMill 4 homogenizer (Fisherbrand). The homogenates were stored at -80°C until further analysis.

**2.3 Extraction of SCFAs from intestinal contents**

For the extraction of SCFAs, 50 µL of sample homogenates (5 mg) in acetonitrile were transferred into an Eppendorf tube (Hamburg, Germany) containing 50 µL of acetonitrile, and 100 µL of 20 µM acetic acid-d4 [internal standard (IS)] in acetonitrile and 100 µL of 20 µM pentanoic acid-d9 (IS) in acetonitrile were added. The prepared mixture was then vortexed vigorously for 3 min at 3,500 rpm at room temperature (RT) and centrifuged (15,000 rpm, 10 min, 4°C), and the supernatant was transferred to a new tube as an SCFA extract.

**2.4 Derivatization of SCFAs with DMED**

To optimize reaction conditions, the DMED-derivatization of authentic SCFAs (5 µM) in the presence of TEA and CMPI was performed under different conditions: 20 µL of TEA, 10 µL of CMPI, and 20 µL of DMED (stock concentrations 0.5–4 mM each) were added

to 100 µL of the standard and 200 µL of the IS mixture and the mixtures were vortexed for 15–60 min at 3,500 rpm at RT–60°C. After centrifugation (15,000 rpm, 10 min, 4°C), 200 µL of the supernatant was transferred to an LC vial for LC-MS analysis as described below. Based on the obtained peak areas, the optimal DMED-derivatization condition was determined as follows; 2 mM for stock concentrations of DEMD, TEA, and CMPI, RT for reaction temperature, and 30 min for reaction duration.

For the DMED-derivatization in the biological samples, 20 µL of TEA (2 mM), 10 µL of CMPI (2 mM), 20 µL of DMED (2 mM), and 200 µL of the IS mixture were added to 100 µL of the SCFA extract containing 5 mg of intestine contents as prepared above. The reaction mixtures were vortexed for 30 min at 3,500 rpm at RT and centrifuged (15,000 rpm, 10 min, 4°C). The DMED-derivatized sample centrifugate (200 µL) was transferred to the LC vials.

**2.5 LC-MS analysis**

Targeted analysis of SCFAs and HSCFAs was performed on a Prominence UFLC (Shimadzu, Kyoto, Japan) connected to TSQ Quantum Mass Spectrometer System (Thermo Fisher Scientific, San Jose, CA, USA) operated in the positive-ion mode. Chromatographic separation was achieved using a Hypersil gold C8 column (50 × 2.1 mm, 5 µm; Thermo Fisher Scientific) maintained at 40°C and mobile phases A: 20 mM ammonium acetate, B: methanol and acetonitrile (1:1) with a flow rate of 0.4 mL/min. Elution gradient as follows: 0–2 min (90% B), 3–5 min (50% B), 6–7 min (20% B). 7.5–10 min (90% B). The sample injection volume was set to 5 µL. The LC-MS/MS was performed using Heated Electrospray Ionization (H-ESI) probe with the following optimized parameters: The DEMD derivatized analytes were directly infused into the triple quadrupole mass spectrometer and MS parameters were optimized for each compound. The schematic representation of DMED derivatization of SCFAs and their ionization pattern are shown in **Figure 1A**. The precursor ion, product ion, tube lens, and collision energies for each of the analytes are described in **Table. 1**. The MS/MS spectra of each of the SCFAs derivatized with DMED are shown in **Figure 1B**. The loss of 45 Da

was commonly observed for all the analytes in their MS/MS spectra. The spray voltage was set to 3500 V, the capillary temperature was 250°C, sheath gas and auxiliary gas were set to 40 and 25 arbitrary units, and the H-ESI vaporizer temperature was set to 150°C. Extracted ion chromatograms and MS spectra were obtained using Xcalibur 2.2 (Thermo Fisher Scientific). The peak area of the analytes was obtained using Xcalibur 2.2 (Thermo Fisher Scientific) software, and their absolute concentrations were calculated from the standard calibration curve.

**2.6 Method validation**

The method validation was performed following the guidelines of previous reports [23, 24]. Stock solutions of each SCFA and HSCFA standard (100 mM) were prepared in acetonitrile solution. The standard stock solutions were mixed and made into a homogenous stock solution containing 1 mM each in acetonitrile. The IS solutions (C2-d4 and C5-d4) were also prepared in acetonitrile at a concentration of 20 μM and kept at −80°C. Three sets of samples containing SCFA and HSCFA standards at three different concentrations: low (25 pmol each), medium (50 pmol each), and high (125 pmol each) were prepared for precision, recovery, and matrix effect validation.

Set A was prepared by spiking the analytes and IS mix solution in acetonitrile. Set B was prepared by spiking the analytes and IS mix solution into the feces samples after extraction (i.e., post-spiked samples). Set C was prepared by spiking the analytes and IS mix solution into the feces samples before extraction (i.e., pre-spiked samples). Five replicates were prepared for each set of samples. Recovery was calculated as the area ratio of the post- to pre-spiked samples (i.e., recovery (%) = set B/set C × 100). The matrix effect was calculated as the area ratio of the post-spiked samples to the acetonitrile samples (i.e., matrix effect (%) = set B/set A × 100). Intra- and inter-day precisions were calculated as the relative standard deviation (RSD) of the spiked analyte and IS peak areas of the feces samples (i.e., RSD (%) = standard deviation/mean × 100). The chemical stability of the analytes was determined from the pre-spiked samples. The stability was calculated by comparing the spiked analyte and IS peak areas of freshly

extracted feces samples with those of samples after 1-week storage at 4 °C. Analyte stabilities were found to be >80%.

### 2.7 Statistics

Statistical analyses were conducted using the GraphPad Prism 8.1 software (GraphPad Software, San Diego, CA, USA). Student's t-tests (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: not significant) were used to detect statistically significant differences between the groups.

## 3. Results and Discussion

Qualitative and quantitative measurements of SCFAs are important to understand their roles in regulating host energy metabolism and immune responses in both physiological and pathological processes. Until now, many methods have been used to analyze SCFAs in the biological samples [8–18]. However, these techniques have problems such as low sensitivity or drastic conditions such as derivatization at high temperatures. Since SCFAs are highly volatile compared to long-chain fatty acids, a novel analyzing method with a very mild condition needs to be established for accurate analyses of SCFAs. DMED, a low molecular weight reagent, is known to react with a carboxylic acid moiety under mild conditions to form amide which enables efficient mass ionization of fatty acids. Past studies by Feng Y-Q *et al*. demonstrated several applications of the DMED reagent for the quantitative analyses of various bioactive molecules [19, 21, 25, 26]. In this study, SCFAs and their hydroxy derivatives were derivatized with DMED under various conditions in terms of reaction temperature (RT to 60°C) and concentrations of DMED, CMPI, and TEA (0.5 to 4 mM) to find the optimal condition to obtain DMED-SCFAs with maximum yield (**Figure 2**).

We first investigated the impact of reaction temperature from RT to 60°C on the derivatization of SCFAs and found that at the ambient temperature, the SCFAs are derivatized with DMED efficiently. Optimal concentrations of DMED, CMPI, and TEA for the derivatization were examined in the range of 0.5–4 mM of each reagent, and it was

found that the reaction with all agents at 2 mM resulted in better intensity and peaks in the LC-MS. On the other hand, when the concentration of the derivatizing agents exceeded 2 mM, the peak of the analyte became unstable, and the signal intensity detected by the LC-MS system decreased. After optimization for reaction temperature and concentrations of reagents, we also checked the derivatization time from 15 to 60 min and determined 30 min for derivatization time based on obtained intensities of DMED-SCFAs in LC-MS. Therefore, the derivatization of SCFAs and their hydroxy derivatives with DMED, CMPI, and TEA at concentrations of 2 mM each for 30 min at RT was selected as the optimized condition for further experiments.

After the optimization of DEMD derivatization conditions and single reaction monitoring channels for each SCFA and HSCFA, the analytical validation was performed using mouse feces as a matrix. For chromatographic separations we have tested Shim-Pac Scepter C18 (100 × 2.1 mm, 5 μm; Shimadzu), Atlantis T3 C18 (2.1×150 mm, 3 μm, Waters, Milford, MA, USA), and Hypersil gold C8 column (50 × 2.1 mm, 5 μm; Thermo Fisher Scientific) columns and found the best peak shapes in the later one. Gradient including isopropanol, methanol, acetonitrile, and milli-Q were used, but significantly improved peak shapes were detected with 20 mM ammonium acetate and methanol: acetonitrile (1:1). However, the study limits the peak resolution of SCFAs and very few molecular species of great importance are targeted. The linearity for each standard was examined for a concentration range of 0.005 to 5000 pmol/μL Linear regression correlation coefficients were greater than 0.99. The calibration curves were obtained from the area ratio of analytes to the IS vs concentration, and the calibration curves of C2 (IS C2-d4) and C5 (IS C5-d9) were used for the calculation of concentrations for analytes with the C2–C3 and C4–C6 carbon chain, respectively. The limit of detection (LOD) in an S/N above 3; the limit of quantitation (LOQ) in an S/N above 10 were determined. The LOD and LOQ for SCFAs are 0.5 and 5 fmol, respectively. These results suggest the method is comparatively highly sensitive to the methods previously reported (LOD/LOQ: 0.1 to 5 pg/ 0.1 -10 pg and 1 to 7 pg/ 3-19 pg respectively) [9, 14]. However,

less sensitive compared to the methods using isotopic labeling coupled LC-MS (LOD: 0.005-0.4 pg and LOQ: 0.015-0.8 pg) and O-benzylhydroxylamine derivatization (LOQ: 0.5-1 fmol) analysis of SCFAs [16, 27]. There is a possibility that the use of isotopically labeled DMED reagent could improve the sensitivity by several folds.

The recovery, matrix effect, and intra- and inter-day reproducibility were evaluated at three different concentrations of each SCFA and HSCFA, and the results are summarized in **Table 2**. The recovery for most of the standards was greater than 80%. The matrix effect determination showed ion suppression (<100%) for most of the species, except for C6, 4HC5, and 3HC6 with ion enhancement (>100%). The extracted ion chromatograms of authentic standards and those detected in samples are shown in **Figure 3**. The elution was completed within a period of 2.5 min. The validated method was then applied for the quantitative analysis of SCFAs and HSCFAs in intestinal samples from mice.

Although the microbiota-derived SCFAs affect host biological processes in the gut, recent studies have indicated diverse roles of SCFAs in other tissues in regulating inflammatory responses which are associated with the progression of type 2 diabetes and nonalcoholic fatty liver disease [6, 7]. Furthermore, viral infections in respiratory tissues have been demonstrated to disturb lipid metabolism in gastrointestinal tracts. Particularly, past studies have demonstrated that the influenza virus infection leads to the reduction of SCFAs in intestinal tissues, which results in the impairment of the gut barrier properties to increase susceptibility to bacterial infections [28, 29]. These findings clearly suggest a potential link between host health and SCFAs derived from gut-microbiota. Actually, many studies have reported that SCFAs protect against viral infections by influencing directly the growth of pathogens or indirectly via the host immune system as summarized in a review article [30].

To understand the kinetic changes in SCFA levels in the intestine during the time-course of severe influenza, in this study, we quantitatively analyzed concentrations of SCFAs in intestinal contents of mice infected with the PR8 virus at 500 PFU as already

reported [22]. At 1, 3, or 6 dpi, mice were sacrificed, and their colon and cecum contents were collected. The results of analyses of SCFA detected in the cecum and colon contents are shown in **Figures 4** and **5**, respectively. In the cecum contents, the concentrations of C2 and C3 fatty acids were not changed significantly after infection, however, C4, C6, and 3HC3 fatty acids were significantly reduced at 3 and 6 dpi in mice infected with the PR8 virus. The C5 SCFA and 3HC4 showed a slight but significant increase at 1 and/or 3 dpi, but not 6dpi. In colon contents, at 1 dpi, no significant changes were observed between the control and PR8 groups. However, at 3 dpi most of the measured SCFAs (C2, C3, C4, C5, 3HC4, and 3HC6) were decreased significantly in PR8 virus-infected mice. At 6 dpi C2, C4, and 3HC6 were significantly decreased in the PR8 group compared to controls whereas the 3HC4 concentration was elevated. These changes suggest the virus infection in the respiratory tissue has a suppressive effect on the lipid metabolism by gut microorganisms producing SCFAs. Moreover, these results are similar to those in previous studies representing the reduction of cecal concentrations of SCFAs (C2, acetate; C3, propionate; and C4, butyrate) after infection with an influenza virus, although acetate and propionate did not decrease in this study [28, 29]. This discrepancy may have been attributed to differences in experimental conditions, such as virus strains, infection titers, and the timing of sample collection. On the other hand, both our results and theirs highlighted the reduction of butyrate in the cecum of mice after day 3 of infection with influenza viruses. Therefore, decreased butyrate could be used as a biomarker for severe influenza as already suggested by a previous study representing a reverse correlation between the abundance of butyrate-producing microbiota in the gut and respiratory viral infection risk in humans [31]. In addition, given the capability of butyrate of promoting differentiation of regulatory T cells [32], a potential role of butyrate in the pathogenesis of or protection against influenza also should be noted. The decreased SCFAs in the colon content could be attributed to the increased transformation into lipids such as short-chain fatty acid esters of hydroxy fatty acids (SFAHFAs) since SFAHFA levels are significantly elevated in the colon contents after

influenza virus infection [33]. Interestingly, 3HC4 increased both in the cecum and colon, which may contribute to the increase of 3HC4 in the circulation, leading to ketoacidosis. However, the biological significance of and the mechanisms behind the influenza-associated changes observed here need to be further investigated. Further, this study limits the information on the interference of isomeric SCFAs, especially when analyzing colon and cecum contents.

We here established the quantitative analyzing method of SCFAs using LC/MS, following the derivatization of SCFAs with DMED under a mild condition so that SCFAs can be efficiently ionized in the positive mode. Method validation was performed with mouse feces as a matrix, and the method was confirmed to have good linearity, LOD, LOQ, measuring range, and repeatability. Further, this established method was applied to analyze the kinetic SCFA profiles in colon and cecum contents of mice during severe influenza. Significant decreases in concentrations of SCFAs in the cecum and colon contents were observed in infected mice, suggesting a possibility that the reduction of SCFAs, particularly butyrate, can be used as a biomarker of respiratory viral infections. In conclusion, the SCFA analyzing method developed in this study is simple, rapid, and sensitive, and will be a powerful tool for measuring the concentrations of SCFAs in biological samples, whose function has been increasingly attracting attention concerning human health and disease.

**Conflicts of interest:**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval:

All the animal protocols were approved by the Animal Care and Use Committee of Hokkaido University (approval no. 17-0003).

**Author contribution:**

DG, SGBG: Conceptualization, Methodology, Supervision. YL, DG, SGBG: Formal analysis, Visualization, Writing - original draft. MO, YL: Data curation, Writing - review & editing. HC, S-PH: Supervision, Resources, Writing - review & editing.

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## TABLES

**Table 1.** Optimized analytes MS setting parameters. CE: collision energy.

| Analyte | Q1 | Q3 | CE (V) | Tube lens (V) |
|---|---|---|---|---|
| Acetic acid (C2) | 131.1 | 86.3 | 12 | 45 |
| Propionic acid (C3) | 145.1 | 100.2 | 13 | 48 |
| Butyric acid (C4) | 159.1 | 114.2 | 11 | 52 |
| Valeric acid (C5) | 173.2 | 128.2 | 12 | 53 |
| Hexanoic acid (C6) | 187.2 | 142.2 | 11 | 51 |
| 3-hydroxypropionic acid (3HC3) | 161.1 | 116.2 | 11 | 47 |
| 3-hydroxybutyric acid (3HC4) | 175.1 | 130.2 | 9 | 46 |
| 4-hydroxypentanoic acid (4HC5) | 189.1 | 144.1 | 14 | 54 |
| 3-hydroxyhexanoic acid (3HC6) | 203.2 | 158.1 | 9 | 50 |
| d4-Acetic acid (C2-d4) | 134.1 | 89.3 | 12 | 50 |
| d9-Valeric Acid (C5-d9) | 187.2 | 142.2 | 11 | 51 |

1

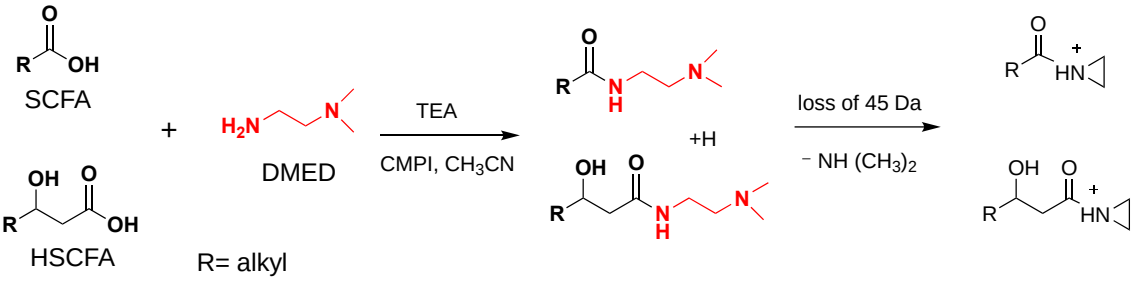
2

**Table 2**. Validation of the method at low (25 pmol), medium (50 pmol), and high (125 pmol) concentrations of spiked standards to feces matrix. The data are shown as mean ± SD, n=5. RSD: Relative standard deviation.

| SCFAs | Recovery (%) | | | Matrix effect (%) | | | Intraday RSD (%) | | | Interday RSD (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Low | Medium | High | Low | Medium | High | Low | Medium | High | Low | Medium | High |
| C2 | 90±3 | 97±2 | 90±4 | 84±8 | 71±8 | 81±5 | 4 | 3 | 3 | 5 | 2 | 4 |
| C3 | 93±4 | 102±7 | 89±3 | 90±6 | 80±7 | 86±2 | 3 | 4 | 4 | 2 | 5 | 2 |
| C4 | 96±2 | 94±2 | 88±1 | 89±3 | 90±12 | 97±7 | 2 | 1 | 2 | 2 | 4 | 2 |
| C5 | 94±3 | 93±5 | 88±2 | 99±6 | 94±2 | 101±6 | 2 | 1 | 3 | 2 | 3 | 2 |
| C6 | 93±4 | 95±5 | 87±3 | 105±10 | 100±2 | 108±8 | 3 | 2 | 2 | 2 | 4 | 2 |
| 3HC3 | 89±3 | 85±1 | 86±2 | 75±4 | 82±5 | 81±3 | 2 | 1 | 4 | 4 | 1 | 2 |
| 3HC4 | 91±6 | 87±3 | 87±3 | 90±6 | 92±3 | 94±2 | 6 | 2 | 4 | 2 | 2 | 2 |
| 4HC5 | 91±2 | 71±2 | 81±1 | 104±3 | 121±2 | 104±3 | 3 | 2 | 3 | 3 | 3 | 3 |
| 3HC6 | 91±3 | 87±3 | 87±1 | 102±5 | 105±4 | 104±5 | 3 | 2 | 3 | 2 | 3 | 1 |
| C2-d4 | 89±4 | 101±7 | 87±3 | 78±5 | 70±6 | 79±2 | 3 | 2 | 4 | 4 | 2 | 3 |
| C5-d9 | 94±2 | 94±4 | 88±2 | 96±5 | 87±3 | 99±8 | 2 | 1 | 2 | 1 | 4 | 3 |

**Figure 1**. **A.** DMED derivatization and ionization patterns of SCFAs and HSCFAs. **B.** The MS/MS spectra of DMED-derivatized SCFAs and HSCFAs. DMED: *N, N*-dimethlyethylenediamine, TEA: triethylamine, CMPI: 2-chloro-1-methyl pyridinium iodide.

**A.**




**B.**

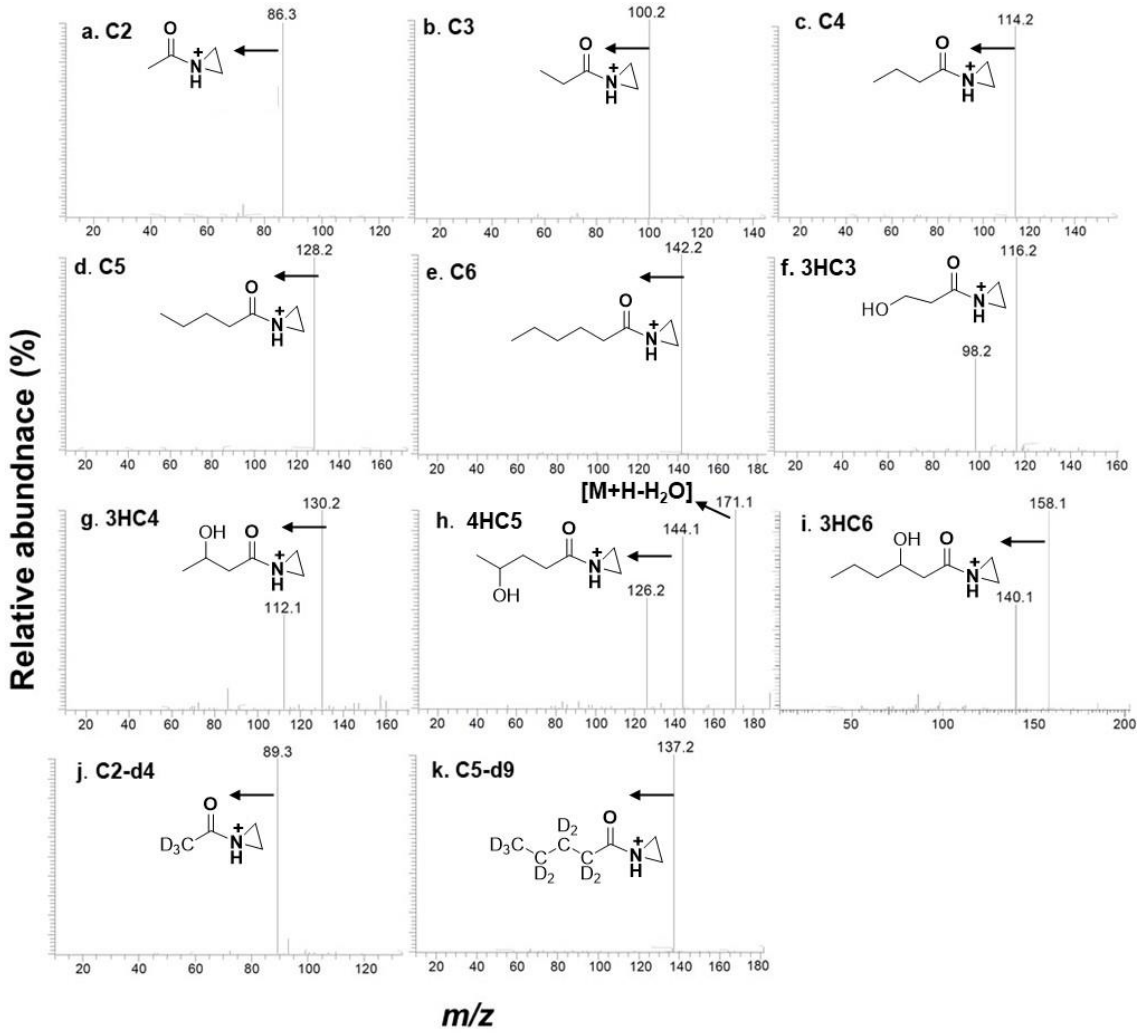


**Figure 2**. Optimization of DMED-derivatization conditions for SCFAs and HSCFAs at various concentrations of CMPI, DMED, TEA and different temperatures

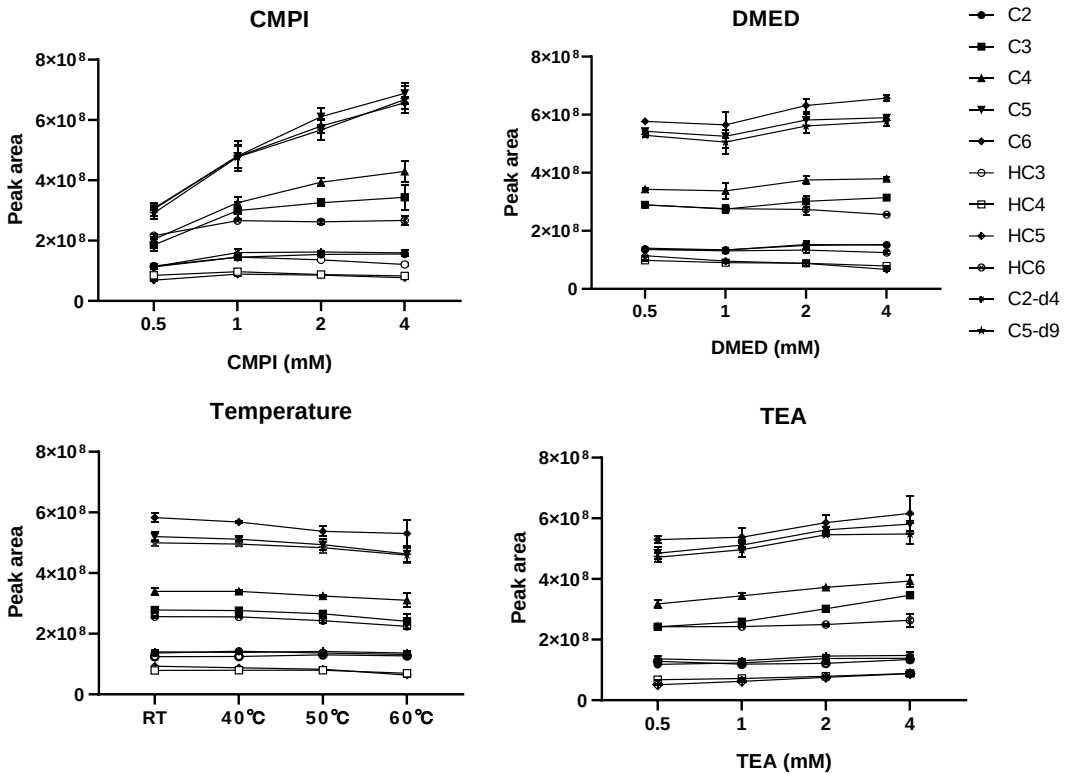


**Figure 3.** Extracted ion chromatograms of SCFAs (A) and HSCFAs (B)- standards vs Samples

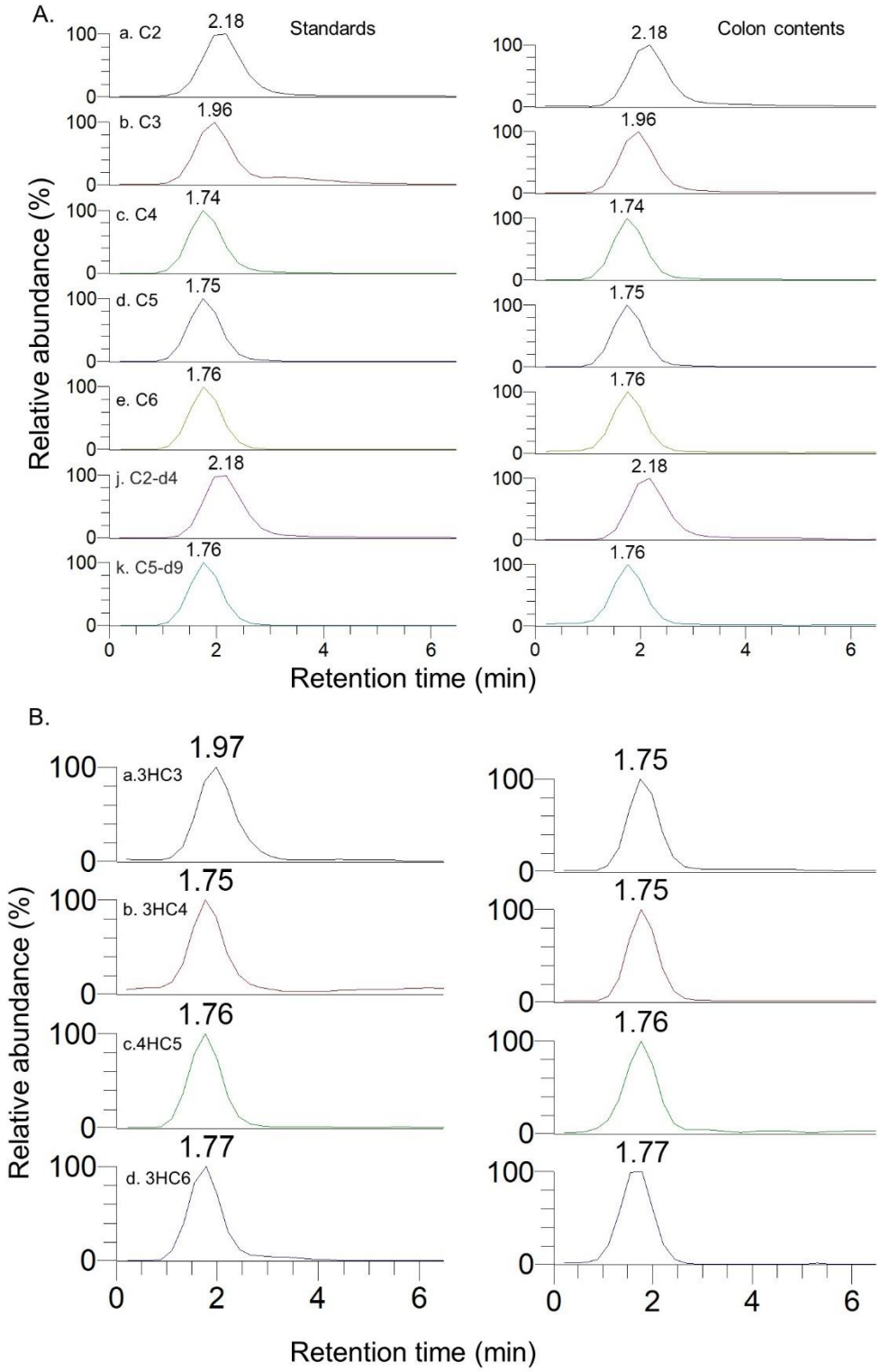


**Figure 4.** Concentrations of SCFAs and HSCFAs detected in the cecum contents of mice (n=5) infected with the influenza virus (PR8). Values are represented by box-and-whisker plots as follows: the central line in the box is the median, the bottom and top lines of the box are the first and third quartiles, respectively, and whiskers are the minimum to maximum values. Student's t-test, Control vs. PR8 at each time point, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. dpi, day-post-infection.

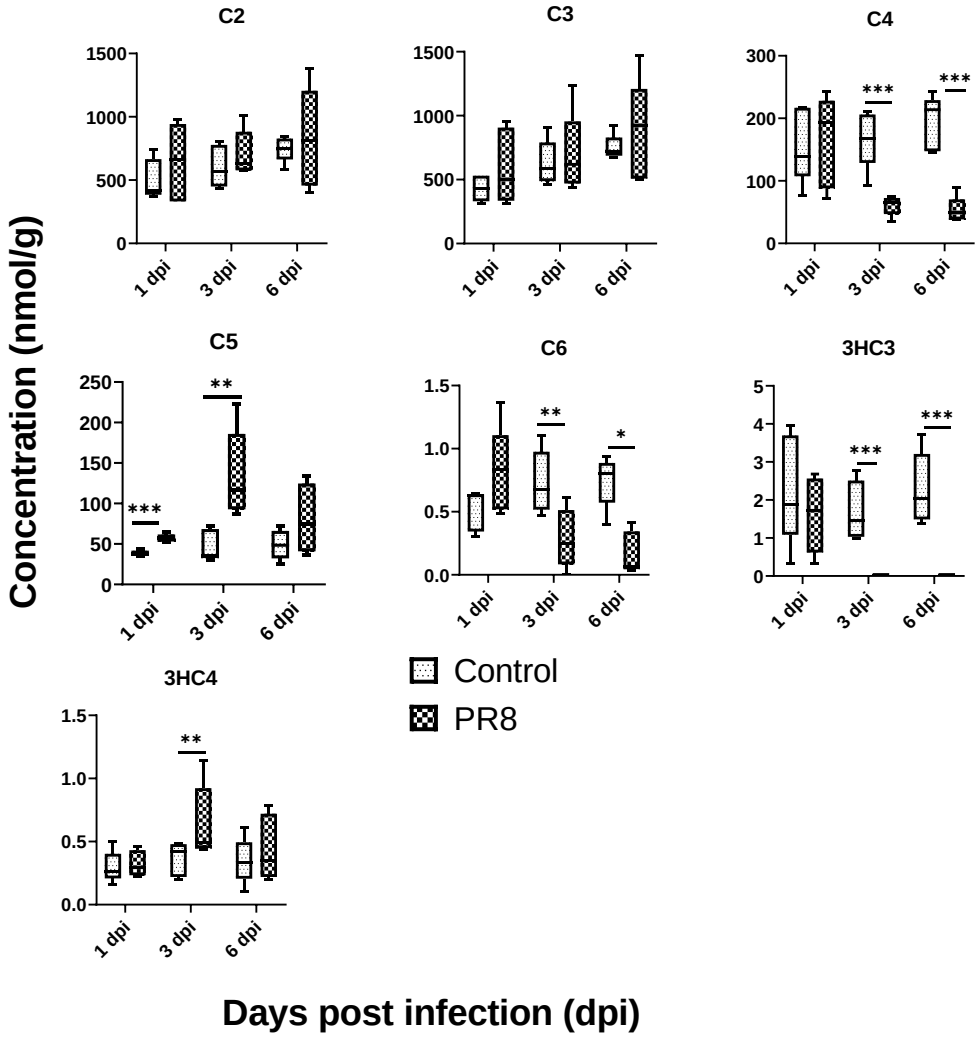
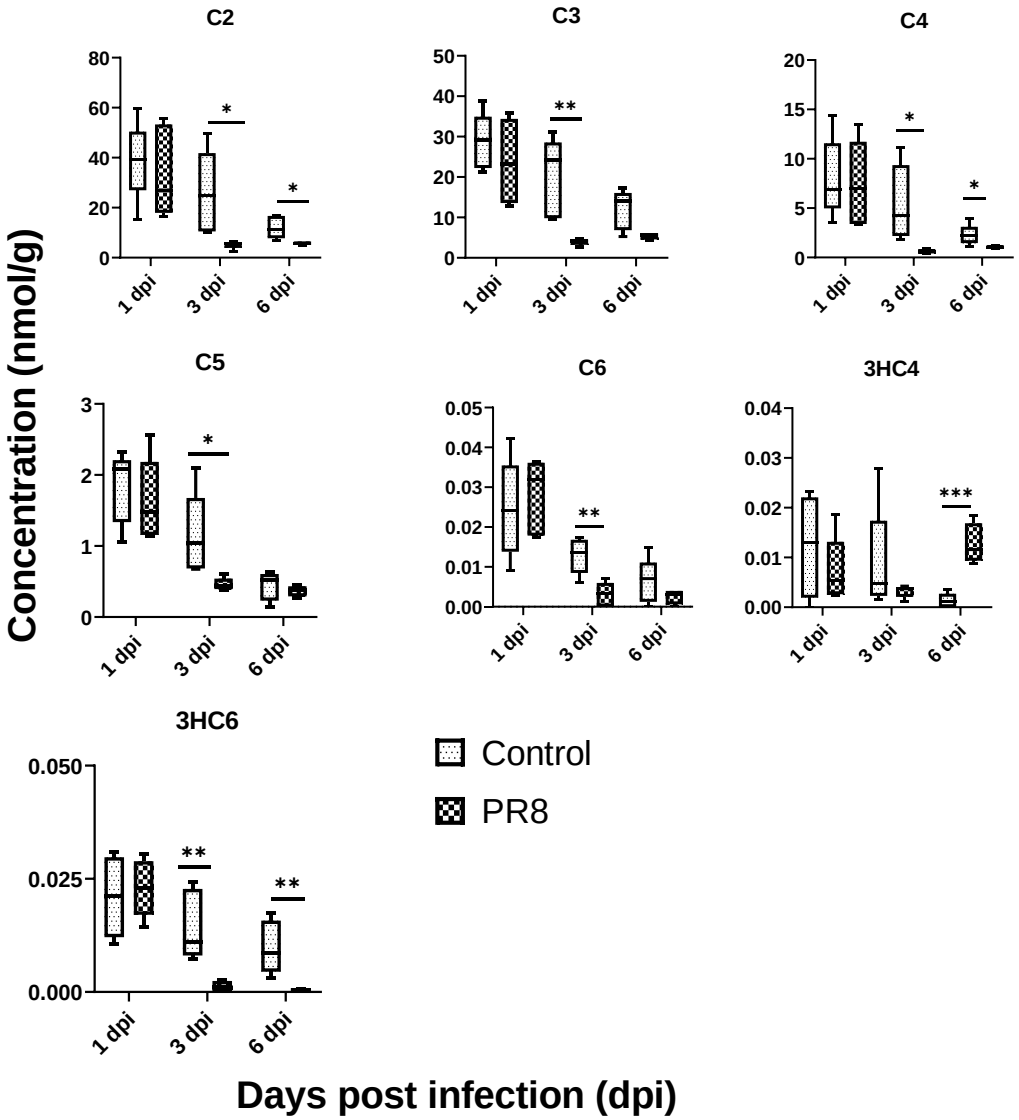


**Figure 5.** Concentrations of SCFAs and HSCFAs detected in the colon contents of mice (n=5) infected with the influenza virus (PR8). Values are represented by box-and-whisker plots as follows: the central line in the box is the median, the bottom and top lines of the box are the first and third quartiles, respectively, and whiskers are the minimum to maximum values. Students t-test, Control vs. PR8 at each time point, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. dpi, day-post-infection.



Graphical abstract